Bacterial communities in a bioelectrochemical denitrification system: The effects of supplemental electron acceptors

Sanath Kondaveeti\textsuperscript{a,1}, Sang-Hoon Lee\textsuperscript{b,1}, Hee-Deung Park\textsuperscript{b,**}, Booki Min\textsuperscript{a,*}

\textsuperscript{a}Department of Environmental Science and Engineering, Kyung Hee University, Gyeonggi-do, South Korea
\textsuperscript{b}School of Civil, Environmental and Architectural Engineering, Korea University, Seoul, South Korea

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\textbf{A B S T R A C T}

Electrochemical treatment of nitrate (NO\textsubscript{3}-) and nitrite (NO\textsubscript{2}-) and mixtures of nitrate and nitrite was evaluated with microbial catalysts on a cathode in three different bioelectrochemical denitrification systems (BEDS). The removal rates and removal percentage of nitrogen (N) compounds varied during biotic and abiotic operations. The biotic cathode using NO\textsubscript{3}-N as an electron acceptor showed enhanced removal percentages (88\%) compared to the operation with NO\textsubscript{2}-N (85\%). The simultaneous reduction of NO\textsubscript{3}-N and NO\textsubscript{2}-N occurred in the operation with a mixture of N compounds. The bacterial diversity from the initial inoculum (return sludge) changed at the end of bioelectrochemical denitrification operation after 55 days. The microbial community composition was different depending on the type of electron acceptor. BEDS operation with NO\textsubscript{3}-N and NO\textsubscript{2}-N was enriched with Proteobacteria and Firmicutes respectively. BEDS with a mixture of N electron acceptors showed enrichment with Proteobacteria. There was no clear, distinct microbial community between the cathode biofilm and suspended biomass.

\textsuperscript{*} Corresponding author. Department of Environmental Science and Engineering, Kyung Hee University, 1 Seocheon-dong, Yongin-si, Gyeonggi-do 446-701, South Korea. Tel.: +82 31 201 2463; fax: +82 31 202 8854.

\textsuperscript{**} Corresponding author. School of Civil, Environmental and Architectural Engineering, Korea University, Anam-Dong, Seongbuk-Gu, Seoul 136-713, South Korea. Tel.: +82 2 3290 4861; fax: +82 2 928 7656.

\textbf{E-mail addresses:} heedeung@korea.ac.kr (H.-D. Park), bkmin@khu.ac.kr (B. Min).

\textsuperscript{1} These authors contributed equally to this work.

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\section{1. Introduction}

Nitrate (NO\textsubscript{3}) and nitrite (NO\textsubscript{2}) have been a worldwide groundwater contaminant mainly due to the use of fertilizers, industrial wastes, animal wastes and septic systems (Szpyrkowicz et al., 2006). High concentrations of nitrate and nitrite in drinking water can cause several health problems such as “blue baby syndrome” for infants, and also can lead to liver damage and cancer in adults (Tannenbaum et al., 1979). The maximum contaminant level of nitrate-nitrogen (NO\textsubscript{3}-N) in water is 10 mg/l in the United States and for nitrite-nitrogen (NO\textsubscript{2}-N) it is around 1 mg/l (Chebotareva and Nyokong, 1997; Park and Yoo, 2009). Various treatment methods such as reverse osmosis (RO), electrodialysis (ED), heterogeneous catalysis (HC), physicochemical, chemical and electrochemical processes have been employed in treating nitrates in water (Virdis et al., 2010). Even though all these methods are employed by choice, they have various disadvantages such as cost, the problem of disposing high saline content and production of toxic byproducts. Besides of all these process
Bioelectrochemical denitrification system (BEDS) using bioelectrochemical processes can be noted as autotrophic denitrification (Huang et al., 2013). Mostly the autotrophic bioelectrochemical denitrification process is considered to be more feasible technology for treating nitrate and nitrite contaminated waters than heterotrophic denitrification. Heterotrophic denitrification is only efficient in nitrate removal when adequate amounts of organic carbon are available (Watanabe et al., 2001). However, in potable water treatment, insufficient organic carbon may limit the application of heterotrophic denitrification unless external organic carbon sources are supplemented. The electrochemical biocathodic reduction process mainly depends upon applied potential and the type of microorganism involved in the denitrification process (Till et al., 1998).

Although bacterial communities associated with heterotrophic denitrification have been well established, information about the microorganisms responsible for cathodic bioelectrochemical denitrification of either nitrate or nitrite is limited. Therefore, in this study, we conducted a bioelectrochemical denitrification process using nitrates, nitrites and a mixture of nitrates and nitrites with the same operational conditions, and an analysis of the bacterial biofilm community for each operation were performed. A pyrosequencing technique was used for monitoring the 16S rRNA gene for possible recovering of uncovered species as in previous studies (Hamady et al., 2010). Based on the data generated from pyrosequencing in this study, we hope to present a detailed analysis of the community structure of cathodic denitrifying bacteria and an analysis of the phylogenetic affiliations corresponding to cathode denitrification in the presence of different nitrogen (N) compound. Electrochemical techniques like cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were also carried out to better understand the factors that limit biocathodic nitrate reductions of different nitrogen ions.

2. Materials and methods

2.1. Bioelectrochemical system set-up

BEDS were operated simultaneously in three different reactors with variation of N compounds such as NO3, NO2 and mixture of NO3 and NO2. The bioelectrochemical system was an “H” type two-compartment cell with a total volume of 270 ml and a working volume of 200 ml for each chamber. Toray carbon paper with a surface area of 22.4 cm² was used as an anode and cathode electrode (Fuel Cell Earth, USA). The electrical connections on the electrode were established using a copper wire. The connections were sealed using a non-conductive epoxy resin (Hardex, USA). A pretreated proton exchange membrane (Nafion 117, Dupont Co., USA) was used to separate the anode and cathode compartments. Membrane pretreatment was carried out as described in previous studies (Kondaveeti and Min, 2013). To prevent membrane swelling when placed in the bioelectrochemical system, membranes were stored in deionized water prior to being used. An Ag/AgCl reference electrode (+0.197 V vs SHE, model: MF-2052, BASi Inc., Korea) was placed in the cathode chamber during the electrochemical analysis. The anode and cathode electrodes were connected to an external DC power supply (Array 3645A, Circuit Specialists Inc., USA) and an external cell voltage of 0.7 V was applied as described previously (Kondaveeti and Min, 2013). All the experiments were carried out in triplicate cycles to observe the consistency in results.

2.2. Reactor inoculum and operating conditions

Initially all the BEDS are operated in abiotic conditions in triplicate. Later the cathode chambers of the reactor were inoculated with return sludge, which was collected from the Giheung Reshipa Wastewater Treatment plant (Yongin-si, Korea). The biofilm formation on the cathode electrode was initialized as described previously (Kondaveeti and Min, 2013). After approximately 25 days of operation (Optimization period) the consistencies in denitrification were observed and further experimental studies have been carried out. During the entire operation (55 days in biotic conditions and 30 days in abiotic conditions), the cathode chambers were fed with a medium containing 100 mM phosphate buffer solution (PBS: NaH2PO4·H2O, Na2HPO4·H2O), 2 g/l NaHCO3, 12.5 ml/l minerals and 5 ml/l trace metal solution (Anderson et al., 2003). 50 mg nitrate-N/l or 50 mg nitrite-N/l was added into the cathode chamber. In the case of nitrate–nitrite reduction test, the cathode chamber was supplemented with both 50 mg nitrate-N/l and 50 mg nitrite-N/l. The biotic and abiotic cathodes differed in the presence of NaHCO3, which was supplemented a carbon source. In entire case the anode chambers for all of the reactors were fed with 100 mM PBS. The reactors were sealed using silicone gel (Wacker Inc., Germany) to ensure strict anaerobic conditions and operated in fed-batch mode. Prior to sealing the reactors, headspace and fluids of both chambers were purged with argon gas (purity >99.9%) for 10 min. Cathode and anode solutions were completely replaced in all the reactors when the concentration of N reached less than 5 mg/l. The solutions were completely mixed by continuously stirring the reactors on a magnetic stirring plate at 160 rpm (ATL-4200, Anytech Co., Korea). BEDS were operated in a temperature controlled incubator (VS-1203P1, Vision Inc., Korea) at 30 ± 1 °C. Biotic control experiments were carried out in an open circuit mode by disconnecting power supplier from the system to determine N reductions in the absence of external power source.
2.3. Analytical procedures

2.3.1. Chemical analyses
From the bio-electrochemical denitrification system, liquid samples were collected for nitrate-N (NO₃⁻-N), nitrite-N (NO₂⁻-N) and ammonia-N (NH₃⁻-N) analysis at every 48 h, starting from the 0th h to the end of operation (240 h). The samples were withdrawn using a 10 ml disposable syringe (Kovax, Korea) and immediately filtered by using an Acrodisc Syringe Filter (0.2 µm HT Tuffryn Membrane). The samples were stored at 4 °C and analyzed using Standard Methods (APHA, 2005) within five days of collection.

2.3.2. Electrochemical analyses
During operation, performance of the bioelectrochemical denitrification system was assessed by employing electrochemical techniques like cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). All electrochemical assays were performed with a three electrode system using a potentiostat (Versa Stat 3, Princeton Applied Research, USA). In BEDS anode and cathode electrode served as a counter and working electrode respectively. An Ag/AgCl electrode was placed near the cathode electrode and used as a reference electrode. The EIS measurements were carried out in a frequency range of 5 m Hz to 100 kHz with an altering AC voltage of 10 mV at a formal open circuit potential. The CV analyses were carried out by sweeping the cell in a potential range of ~0.8 V to 0.6 V with a scan rate of 5 mV/s. This potential range (~0.8 V~0.4 V) was successfully used in the other studies carried out by Su et al. (2012).

2.4. Microbial community analysis

2.4.1. Biomass sampling, DNA extraction, PCR and pyrosequencing
To compare the differences in bacterial communities of each BEDS, biomass samples of day 0 and 55 cathode mixed liquor and day 55 cathode biofilm, were collected. For the biofilm sample, the cathode sheets were rinsed with sterilized water three times and were cut into ~20 pieces (total area approx. = 1 cm²) for the extraction of biofilm community DNA. DNA was extracted from the biomass samples (in duplicate) using the MoBio PowerSoil DNA extraction kit (Solana Beach, CA, USA) according to the manufacturer’s protocol. The concentration and purity of the extracted DNA were determined using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and were stored at ~20 °C until pyrosequencing. The typical concentration and purity (Abs. 260 nm/ Abs. 280 nm) of the total DNA extracted from the samples were around ~50 ng/µL and ~1.9, respectively. For the pyrosequencing, bacterial 16S rRNA genes were amplified by using 27F (5‘-AGAGTTTGATCMTGGCTCAG-3’) (Massol-Deya et al., 1995) and 518R (5‘-ATTACCGCGGCTGCTGG-3’) (Lane, 1991) primers targeting the V1, V2 and V3 hypervariable regions of the bacterial 16S rRNA gene. All of the pyrosequencing procedures including construction of a single-stranded DNA library, emulsion PCR and a pyrosequencing reaction were conducted at Macrogen (Seoul, South Korea) as described in our previous study (Kwon et al., 2010).

2.4.2. Analyses of sequence data
The 16S rRNA gene sequences obtained from pyrosequencing was initially sorted by keys using a customized Perl script program, named as “trimBarcode.pl” (Macrogen). Using this program, the adapter sequences, low quality (<Q20) and short (<300-bp long) sequences were trimmed. Potential chimeric sequences were also discarded using the chimera UCHIME algorithm in Mothur utility (Schloss et al., 2009). Taxonomic classification of the sequences was conducted using the RDP’s Classifier (Cole et al., 2005). Diversity indices such as observed Operational Taxonomic Unit’s (OTUs) richness, Chao1 richness estimator and abundance-based coverage estimator (ACE) were determined by the Mothur utility (Schloss et al., 2009). All of these procedures were followed according to the directions of the RDP Pyrosequencing Pipeline (http://pyro.cme.msu.edu/) (Kwon et al., 2010; Lee et al., 2013).

2.5. Statistical analysis
To compare the composition of bacterial communities within and between each BEDS sample, principal component analysis (PCA) was performed using Canoco version 4.5 (Plant Research International, Wageningen, Netherlands) according to the manufacturer’s manual. PCA is an ordination technique widely used for providing a visual determination of the similarities among samples in community data (Chen et al., 2008). PCA analysis was carried out based on the OTU composition.

2.6. Phylogenetic analysis
In order to assess the phylogenetic affiliation of core OTUs observed in this study, a phylogenetic tree was inferred using a neighbor-joining algorithm. For construction of the tree, closely related sequences of core OTUs (i.e., reference sequences) were collected from the RDP database and GenBank database using the RDP’s Sequence Match (Cole et al., 2009) and the NCBI BLAST (Altschul et al., 2009, 1997), respectively. The core OTUs and the reference sequences were aligned using CLUSTAL-W (Thompson et al., 1994), and were used to construct a phylogenetic tree. The relative evolutionary distances among sequences were calculated by using the Kimura 2-parameter model (Kimura, 1980) and the tree topology was evaluated statistically by 1000 bootstrap resamplings. All of the procedures for constructing the phylogenetic tree were conducted by using MEGAS5 software (Kumar et al., 2008).

3. Results and discussion

3.1. Electrochemical denitrification of N compounds in biotic operation
During the 240 h biotic operational cycle, a total of 50 mg/l NO₃⁻-N was reduced to 6 mg/l NO₃⁻-N with a reaction rate of 0.204 mg:NO₃⁻-N/cm² d, with a removal percentage of 88% (Fig. 1). During the degradation process of nitrate, intermediate products like nitrite and ammonia were observed in the solutions. At 240 h of operation, nitrite and ammonia were accumulated at a concentration of 4.5 mg/l NO₂⁻-N and 0.5 mg/l NH₃⁻-N, respectively. Similar intermediate product formation
was observed in other studies of bioelectrochemical denitrification systems (Dhamole et al., 2007). In other studies of the denitrification process, only 58% nitrate removal was observed for 192 h of operation using the anode as an electron source for nitrate reduction (Jia et al., 2008). Park et al. (Park et al., 2005) has operated a similar biofilm-electrode reactor and obtained a maximum removal rate of 0.171 mg NO₃-N/cm² d.

The reactor fed with only nitrite showed a removal percentage and removal rate of 85% and 0.198 mg NO₂-N/cm² d (10.6 g NO₂-N/m³ d), respectively (Fig. 1). In this reactor, the initial 55 mg NO₂-N/l was reduced to 8 mg NO₂-N/l. No nitrite oxidation to nitrate was observed in the cathode chambers, whereas the ammonia concentration was found to be less than 0.7 mg/l. In other studies of autotrophic denitrification, an initial 42 mg/l NO₂-N was reduced to 20 mg/l NO₂-N with a removal rate: 7.6 g NO₂-N/m³ d during the 4 h continues mode of operation with an anode as an electron source (Puig et al., 2011).

For nitrites, lower removal rates compared to nitrate-nitrogen have been observed because of the slow electron acceptance capacity. Also, it is not a choice electron acceptor for denitrification due to the differences in enzyme activity (Williams et al., 1978). This can be considered as one of the limiting factors during the cathodic electrochemical denitrification process.

The reactor containing the mixture of nitrate and nitrite showed a removal percentage of 84% with a removal rate of 0.188 NO₃-N/cm² d for nitrate. In this reactor, the initial 50.4 mg/l NO₃-N was reduced to 8 mg/l NO₃-N. On the other hand, another N compound, i.e., nitrite, showed a reduction of initial 53.7 mg/l NO₂-N to 6.5 mg/l NO₂-N for 240 h of operation (Fig. 1). The nitrite reductions showed a removal percentage and removal rate of 87% and 0.21 mg NO₂-N/cm² d, respectively. These results suggest that the simultaneous reductions of nitrates and nitrites can be achieved in an electrochemical denitrification process. Based on the mass balance, the reactor operating with nitrates showed 78% nitrogen formation. On other hand, the cathodic denitrification system operating with nitrites and a mixture of nitrates and nitrites showed 83% and 61% nitrogen formation, respectively (Table 1).

During the electrochemical process of denitrification at 0.7 V, cathode potential was sustained at around –330 ± 20 mV (vs Ag/AgCl) in all of the reactors. At this potential it was unlikely to produce H₂ gas because hydrogen is generally produced above –0.41 V (abiotically) (Cheng et al., 2012) or because the reduction potential of hydrogen generation requires a theoretical voltage of –0.42 V (Logan, 2008). In addition, no diffusion of N compounds towards the anode chamber was observed and no nitrate reductions were observed in the biotic control experiments in an open circuit mode without external

### Table 1 – Biotic and abiotic electrochemical denitrification of N compound in cathode chamber at an applied voltage of 0.7 V.

<table>
<thead>
<tr>
<th>N compound</th>
<th>Removal rate mg NO₃ or NO₂-N/cm² d</th>
<th>“N” removal percentage</th>
<th>N₂% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biotic operation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃-N</td>
<td>0.204 ± 0.003</td>
<td>87.9</td>
<td>78</td>
</tr>
<tr>
<td>NO₂-N</td>
<td>0.198 ± 0.0094</td>
<td>85.4</td>
<td>83</td>
</tr>
<tr>
<td>NO₃-N and NO₂-N</td>
<td>0.188 ± 0.001(NO₃)</td>
<td>84.7 (NO₃)</td>
<td>61 (NO₃ + NO₂)</td>
</tr>
<tr>
<td>NO₃-N and NO₂-N</td>
<td>0.210 ± 0.002(NO₂)</td>
<td>87.7 (NO₂)</td>
<td></td>
</tr>
<tr>
<td><strong>Abiotic operation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO₃-N</td>
<td>0.163 ± 0.0007</td>
<td>74.8</td>
<td>60</td>
</tr>
<tr>
<td>NO₂-N</td>
<td>0.191 ± 0.0017</td>
<td>78.1</td>
<td>70</td>
</tr>
<tr>
<td>NO₃-N and NO₂-N</td>
<td>0.125 ± 0.0002(NO₃)</td>
<td>57.1 (NO₃)</td>
<td>40 (NO₃ + NO₂)</td>
</tr>
<tr>
<td>NO₃-N and NO₂-N</td>
<td>0.142 ± 0.0003(NO₂)</td>
<td>64.5 (NO₂)</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated based on the mass balance.
power source. Therefore, the observed cathodic denitrification was most likely carried out by autotrophic denitrifying biofilm by using the cathode electrode as the sole electron source (Cheng et al., 2012). However, there could have been the possibility of a small amount of abiotic nitrogen reduction during the bioelectrochemical reactions (Kondaveeti and Min, 2013). We observed from the abiotic denitrification process that nitrates and nitrites showed removal rates of 0.178 mg NO\textsubscript{3}\,-N/cm\textsuperscript{2} d\textsuperscript{-1} and 0.191 mg NO\textsubscript{2}\,-N/cm\textsuperscript{2} d\textsuperscript{-1}, respectively. The reactor with a mixture of nitrates and nitrites showed a removal rate of 0.125 mg NO\textsubscript{3}\,-N/cm\textsuperscript{2} d\textsuperscript{-1} and 0.145 mg NO\textsubscript{2}\,-N/cm\textsuperscript{2} d\textsuperscript{-1}, respectively. These nitrogen removal rates during abiotic operation were lower in comparison with the biotic operation regardless of the type of N compound (Fig. S1).

3.2. Analyzing the internal resistances of the cathode electrode

The internal resistances of the cathode electrode with different N containing compounds were analyzed by electrochemical impedance spectroscopy (EIS). The impedance of the working electrode elucidates rate limiting factors in the denitrification process by pinpointing the internal resistance ($R_{\text{in}}$) of the system. The total internal resistances of the system were dominated by charge transfer resistance; this can be decreased by developing the biofilm on the cathode electrode. In all three cases, less internal resistances were observed compared to the abiotic cathode (Fig. S2). This indicates that the development of biofilm on the cathode electrode was established (He and Mansfeld, 2009). The reactor containing either nitrate or nitrite showed a similar ohmic resistance around 3 $\Omega$ (Fig. 2). Dominant internal resistance, i.e., the charge transfer resistance, was found to be around 150 $\Omega$ and 160 $\Omega$ with nitrate and nitrite, respectively. The reactor operating with a mixture of nitrate and nitrite showed an ohmic resistance of 7.2 $\Omega$ and a charge transfer resistance of 200 $\Omega$. This result suggested that the higher charge transfer resistance was one of the major limiting factors during the bioelectrochemical reduction process. The different charge transfer resistances might be due to the different biocatalysts formed on the cathode depending on the type of N containing chemicals. As shown in Fig. 2, the cathode impedance was primarily composed of charge transfer resistance, which is inversely proportional to the exchange current density (Manohar et al., 2008). The exchange current densities ($I_{\text{ex}}$) were calculated by the following equation (Borole et al., 2010):

$$I_{\text{ex}} = \frac{\text{RT}}{nF} \frac{1}{R_{\text{ct,-cathode}}} \times \frac{1}{A_{\text{cathode}}}$$

In the above equation, $R$ is known as the gas constant in joules per mole Kelvin, $T$ is for temperature in Kelvin, $n$ is number of electrons involved in the charge transfer reaction ($n = 5$ assuming that nitrate was converted to nitrogen and assuming $n = 2$ for conversion of nitrite to nitrogen), $F$ is the Faraday constant in coulombs per mole and $A$ is area of the cathode electrode. The maximum cathode exchange current density ($I_{\text{ex}}$) was 0.0145 A/m\textsuperscript{2} with nitrate and the reactor operating with nitrite showed an $I_{\text{ex}}$ of 0.026 A/m\textsuperscript{2}. The exchange current operating with a mixture of both nitrite and nitrate was 0.0124 A/m\textsuperscript{2} and 0.0244 A/m\textsuperscript{2} with nitrate and nitrite, respectively.

**Fig. 2** – Representative Nyquist curves for three different biocathode reactors operating with NO\textsubscript{3}\,-N (A), NO\textsubscript{2}\,-N (B) and mixture of NO\textsubscript{3}\,-N and NO\textsubscript{2}\,-N (C). The black square line (■) is without “N” compound and red square line (●) is with “N” compound. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 3** – Cyclic Voltammograms corresponding in three different biocathode reactors operating with NO\textsubscript{3}\,-N (A) NO\textsubscript{2}\,-N (B) mixture of NO\textsubscript{3}\,-N and NO\textsubscript{2}\,-N (C). The black square line (■) is without “N” compound and red square line (●) is with “N” compound. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. Cyclic Voltammograms of the biocathodes with three different compositions of nitrogen ions

The electrochemical characteristics of the three biocathodes with nitrate and nitrite were elucidated by using the electrochemical technique cyclic voltammetry (CV) (Fig. 3). Cyclic voltammetry is a highly used electrochemical technique that helps to characterize and evaluate the redox activities of components during fuel cell operation (Mohan and Chandrasekhar, 2011). Cyclic voltammetry visualized significant variation in the redox peaks and electron transfer mechanism (the potential at which the reduction reaction takes place) depending upon the microbial enzymes involved in the redox reactions on the electrode and the redox chemicals (i.e., NO₃ and NO₂) in the cathode chambers of the reactor. No reduction peaks were observed in CV for all three of the cathodes in absence of a N compound. By injecting nitrate or nitrite into the cathode chamber, the reduction peaks were observed at different potentials depending upon the compounds. The denitrifying biocathode with only nitrate showed a reduction peak at −130 mV vs. Ag/AgCl with a peak current I of −1.8 mA. For nitrite, the reduction peak was observed at −283 mV with a peak current of −0.061 mA. Two reduction peaks were observed in the case of the reactor containing a mixture of both nitrite and nitrate, the reduction peaks was observed at −570 mV (nitrite) and −260 mV (nitrate) with a peak current of −5.58 mA and −4.07 mA. For the nitrite and nitrate reactor, the oxidation peak was observed at 110 mV, assuming that the oxidation peak observed was due to the conversion of nitrite to nitrate. In other studies of denitrification with mixtures of nitrate and nitrite, a similar oxidation peak was observed at −0.47 mV vs SCE (Manea et al., 2010). No oxidation peaks were observed for the reactors operating with only nitrite or nitrate.

3.4. Bacterial community structure of the BEDSs supplemented with different electron acceptors

From the eight biomass samples of the BEDS, a total of 18,116 good quality sequences (805–2166 sequences) were retrieved, after removing both <300 bp sizes of sequences and chimeric sequences (Table 2). The average number of sequences was 446.3 ± 46.6 bp (311–541 bp). The highest numbers (2166 sequences) of sequences were retrieved from the day 55 biofilm sample supplemented with NO₃/NO₂, while the lowest numbers (805 sequences) were retrieved from the day 55 mixed liquor sample supplemented with NO₂.
sequences were assigned taxonomically using the RDP’s Classifier. Sequences estimated with <80% bootstrap cutoff were assigned to unclassified sequences. A total of 23 phyla were analyzed from 18,069 sequence reads.

Fig. 4 shows the bacterial community compositions of seed biomass (i.e., day 0 mixed liquor) and day 55 biofilms and mixed liquor samples. In the seed biomass, Bacteroidetes (41.3 ± 3.1%), Proteobacteria (14.5 ± 0.2%) and Acidobacteria (11.3 ± 2.3%) were the three major phyla. During 55 days of operation, each B Edwards experienced significant changes in the bacterial composition. At day 55, each cathode biofilm sample demonstrated a unique composition depending on the supplemental electron acceptor. Firmicutes (50.5 ± 4.4%) was the predominant phylum in the NO2-supplemented system, while Proteobacteria (42.5 ± 3.9%) and Bacteroidetes (36.7 ± 1.0%) were the two major phyla in the NO3-supplemented system. In the NO2 + NO3-supplemented system, Proteobacteria (85.5 ± 8.4%) showed the highest proportion. Among the proteobacterial sequences, the proportion of class Gamma proteobacteria was highest in all of the samples, except for the day 0 mixed liquor sample. At day 55, each mixed liquor sample showed a similar community composition with its cathode biofilm sample. There was no statistical difference between the cathode and the mixed liquor samples (P > 0.280, ANOVA). These results suggest that the different operational conditions (i.e., different supplemental electron acceptors) selected specific bacterial populations adapted to the condition. In addition, the similar compositions between the biofilm and mixed liquor samples may imply that the 55 days of operation was not sufficiently long to generate significant differences in the bacterial communities.

The bacterial diversity significantly decreased during the 55 days of operation in each B Edwards (Table 3). The highest numbers of OTUs were detected in the day 0 mixed liquor samples (743 ± 8.5). At day 55, the number of OTUs decreased to 278 ± 136.0 and 214 ± 14.2 for biofilm and mixed liquor, respectively. These results suggest that the operational conditions affected the diversity as well as the community compositions.

The bacterial communities of B Edwards reactors were further compared based on similarities of OTUs using PCA. In the PCA ordination shown in Fig. 5, the samples of cathode biofilm and mixed liquor samples for the same electron acceptors were clustered together, which suggested that the bacterial community compositions were similar when electron acceptors were the same. On the other hand, the clusters were located separately in the ordination by the electron acceptor, indicating that the community compositions were structured differently by the supplemented electron acceptors. These results are in line with the analyses of bacterial communities by phylum seen in Fig. 4.

3.5. Phylagenetic association of the core bacterial OTUs in each B Edwards

To identify the bacterial phylotypes observed in the B Edwards in detail, a phylagenetic tree was constructed using 17 core bacterial OTUs with their closely related sequences (Fig. 6). The core bacterial OTUs were defined as the OTUs greater than 3% of the total sequence reads in each sample. Seventeen OTUs, out of 2552 OTUs, were assigned to the core bacterial OTUs that constituted 29.1% of the total sequence reads. Among the 17 core bacterial OTUs, the sequences belonging to the phyla Bacteroidetes and Proteobacteria were the most abundant ones (6 OTUs each), followed by Firmicutes (3 OTUs), Chloroflexi (1 OTU) and Deinococcus-Thermus (1 OTU).
Four of the 6 core OTUs (OTU 173, 189, 216 and 258) belonging to the phylum Bacteroidetes were most frequently found in the biofilm and mixed liquor samples of the BEDS supplemented with NO₃/C₀ (0.7%–17.8% and 0.2%–27.9% of the sequences, respectively), while the sequences within OTU 1 and 48 were found at the highest proportion in the day 0 mixed liquor (3.3–3.7% of sequences). Among these (6 OTUs), the sequences within OTU 173 were mostly distributed in the

<table>
<thead>
<tr>
<th>Names of sample</th>
<th>No. of OTUs</th>
<th>No. of sequences</th>
<th>Richness estimation²</th>
<th>Chao¹&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ace&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>2552</td>
<td>18069</td>
<td>5792 (5357–6295)</td>
<td>9683 (9240–10156)</td>
<td></td>
</tr>
<tr>
<td>Day 0 mixed liquor-1</td>
<td>737</td>
<td>1624</td>
<td>2857 (2331–3557)</td>
<td>5053 (4615–5542)</td>
<td></td>
</tr>
<tr>
<td>Day 0 mixed liquor-2</td>
<td>749</td>
<td>1664</td>
<td>2306 (1948–2772)</td>
<td>5021 (4586–5505)</td>
<td></td>
</tr>
<tr>
<td>Day 35 Cathode biofilm [NO₂] – 1</td>
<td>345</td>
<td>1495</td>
<td>849 (688–1086)</td>
<td>1376 (1208–1578)</td>
<td></td>
</tr>
<tr>
<td>Day 35 Cathode biofilm [NO₂] – 2</td>
<td>512</td>
<td>2059</td>
<td>1915 (1508–2487)</td>
<td>3442 (3087–3846)</td>
<td></td>
</tr>
<tr>
<td>Day 35 Cathode biofilm [NO₃] – 1</td>
<td>186</td>
<td>884</td>
<td>433 (332–604)</td>
<td>806 (648–955)</td>
<td></td>
</tr>
<tr>
<td>Day 35 Cathode biofilm [NO₃] – 2</td>
<td>169</td>
<td>806</td>
<td>318 (257–421)</td>
<td>610 (512–736)</td>
<td></td>
</tr>
<tr>
<td>Day 35 Cathode biofilm [NO₂ + NO₃] – 1</td>
<td>289</td>
<td>2166</td>
<td>655 (533–839)</td>
<td>1269 (1102–1470)</td>
<td></td>
</tr>
<tr>
<td>Day 35 Cathode biofilm [NO₂ + NO₃] – 2</td>
<td>165</td>
<td>1757</td>
<td>508 (350–802)</td>
<td>579 (485–701)</td>
<td></td>
</tr>
<tr>
<td>Day 35 mixed liquor [NO₂] – 1</td>
<td>223</td>
<td>857</td>
<td>482 (385–638)</td>
<td>793 (673–945)</td>
<td></td>
</tr>
<tr>
<td>Day 35 mixed liquor [NO₂] – 2</td>
<td>225</td>
<td>805</td>
<td>515 (408–685)</td>
<td>997 (844–1189)</td>
<td></td>
</tr>
<tr>
<td>Day 35 mixed liquor [NO₃] – 1</td>
<td>211</td>
<td>820</td>
<td>670 (490–967)</td>
<td>1330 (1126–1580)</td>
<td></td>
</tr>
<tr>
<td>Day 35 mixed liquor [NO₃] – 2</td>
<td>227</td>
<td>980</td>
<td>478 (386–623)</td>
<td>804 (685–953)</td>
<td></td>
</tr>
<tr>
<td>Day 35 mixed liquor [NO₂ + NO₃] – 1</td>
<td>207</td>
<td>1100</td>
<td>477 (374–643)</td>
<td>944 (810–1108)</td>
<td></td>
</tr>
<tr>
<td>Day 35 mixed liquor [NO₂ + NO₃] – 2</td>
<td>190</td>
<td>1052</td>
<td>468 (356–656)</td>
<td>727 (613–872)</td>
<td></td>
</tr>
</tbody>
</table>

* Values are calculated at the 97% confidence intervals.

<sup>b</sup> The values indicate average and range (minimum to maximum).

PCA plot

Fig. 5 – PCA ordination of the inoculum of anaerobic digester sludge (0 day), and cathode biofilm (55 day) and cathode mixed liquor (55 day) samples based on OTU compositions.
day 55 samples of the BEDS: 17.8% and 27.9% of each total sequences for biofilm and mixed liquor samples, respectively. However, only limited sequences were detected in the day 0 mixed liquor sample (0.0%). The sequences within this OTU were closely related with a 16S rRNA gene clone (JQ987636) from the BEDS in our previous study (Lee et al., 2013) (identity = 99%). The closest related sequence of the culture strain was that of Weeksella virosa (NR074495) (identity = 86%). OTU 216, which was also frequently detected in the cathode biofilm (6.9% of total sequences), showed the closest identity (94%) with the 16S rRNA gene sequence of Proteiniphilum acetatigenes TB107 (AY742226) isolated from an anaerobic wastewater treatment plant (Zhang et al., 2010). Moreover, they were also closely related with the BEDS clone MEB2-922 (JQ987950) (>98%) from the cathode biofilm samples in our previous study (Lee et al., 2013). However, it is not clear yet whether W. virosa and P. acetatigenes were actively involved in nitrate reduction in situ and whether these strains are able to grow under strictly autotrophic conditions or not. On the other hand, the sequences within OTU 1 and 48 were mostly

![Fig. 6 – Phylogenetic affiliation of 16S rRNA gene sequences of 17 core OTUs with the phylogenetically related sequences obtained from the RDP-II and GenBank database. The genetic distances of each sequence were calculated using the Kimura 2-parameter model and the tree was constructed using the neighbor-joining algorithm. The bootstrap score (numbers at the nodes) are shown for frequencies above the threshold of 50%. The tree was out-grouped with a 16S rRNA gene sequence of Thermococcus barophilus (Archaea outgroup).](image-url)
detected in the day 0 mixed liquor (3.3% and 3.7% of total sequences, respectively) and their distribution decreased during the 55 days of operation.

Five of the 6 core OTUs (OTU 13, 15, 23, 131, 153) were most frequently found in the biofilm and mixed liquor samples from all three of the BEDS. These OTUs were phylogenetically affiliated to the phylum Proteobacteria. Of the OTUs, the sequences within OTU 15 and 23 were closely related with nitrate utilizing bacteria Paracoccus alcaliphilus (AY014177) and Pseudomonas stutzeri (NR074829), respectively. These strains are known to grow under strictly autotrophic conditions (Katayama et al., 1995; Mahmood et al., 2009). They showed the highest proportion in the day 55 biofilm samples of BEDS supplemented with NO$_3$/NO$_2$ (5.5% for P. stutzeri and 0.4% for P. alcaliphilus, respectively), and the day 55 mixed liquor samples supplemented with NO$_3$/NO$_2$ (5.1% P. stutzeri and 6.8% P. alcaliphilus, respectively). In contrast, the sequences within OTU 13, 131 and 153 were closely related to the sequences of Halomonas korensis (EU085033), which is known as both a nitrate- and nitrite-reducing species (Li et al., 2008). They were most frequently distributed in the day 55 biofilm and mixed liquor samples supplemented with NO$_2$. Interestingly, the sequences within OTU 277 were detected only in the day 55 biofilm samples supplemented with NO$_3$. The closest identity of OTU 277 was the sequence of Thauera aromatica (X77118), which is known as a nitrate-reducing microorganism (Anders et al., 1995). Interestingly, all of the proteobacterial core OTUs monitored in this study are known to possess a nitrite and/or nitrate utilizing function, although the distribution of these OTUs were differentiated by the supplemental electron acceptors. The results suggest that the proteobacterial OTUs might be ubiquitous in BEDS irrespective of the supplemental electron acceptors.

All of the core OTUs classified within the phylum Firmicutes were abundantly found in the day 55 biofilm and mixed liquor samples supplemented with NO$_3$ (5.0–24.5% and 0.3–5.0%, respectively) and NO$_3$/NO$_2$ (1.2–4.5% and 0.3–1.4%, respectively). The closest identify (>96%) of these OTUs was the sequence of Bacillus novulis (AJ542512), which is known to have a nitrate utilization ability (reducing nitrate to nitrite), but is not able to grow autotrophically under anaerobic conditions (Jeroen Heyrman et al., 2004). However, there have been no publications on the nitrite utilization activities of this bacterium. Considering there was no nitrite reduction activity from this species, OTUs belonging to this phylum are unlikely to be involved in denitrification activity.

4. Conclusion

Simultaneous reductions of nitrate and nitrite were successfully observed in BEDS at an external voltage of 0.7 V. The removal rate of nitrate was a little bit lower in comparison to nitrite as a cathode electron acceptor. The differences in supplementary electron acceptors were reflected in the bacterial community compositions. Nitrate and nitrite supplementation enriched bacteria mostly within Proteobacteria and Firmicutes, respectively for both cathode biofilms and mixed liquors, while nitrate plus nitrite supplementation enriched bacteria within Proteobacteria. The differences in bacterial community compositions were confirmed by PCA ordination and phylogenetic analyses. Microbial compositions on the BEDS cathode and in the bulk solution during the 55 days of operation were similar except for a difference in the number of OTUs. This study provides a better understanding of the bioelectrochemical denitrification process and the responsible bacterial communities in the removal of different nitrogen ions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.12.023.

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